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Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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RNA virus-derived plant expression system

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RNA VIRUS-DERIVED PLANT EXPRESSION SYSTEM

FIELD OF THE INVENTION

The present invention relates to a viral vector system for replicating or for expressing a sequence of interest in a plant. The invention also provides a process for replicating and/or expressing a sequence of interest in a plant. This process can be used for expressing a protein of interest in plants, notably in crop plants. The system can be based on a large variety of different viral vectors.

BACKGROUND OF THE INVENTION

Virus-based expression systems can be used for rapid protein production in plants (for review see: Porta & Lomonossoff, 1996, *Mol. Biotechnol.*, 5, 209-221; Yusibov *et al.*, 1999, *Curr.Top. Microbiol. Immunol.*, 240, 81-94) and are a powerful tool for functional genomics studies (Dalmay *et al.*, 2000, *Plant Cell*, 12, 369-379; Ratcliff *et al.*, 2001, *Plant J.*, 25, 237-245; Escobar *et al.*, 2003, *Plant Cell*, 15, 1507-1523). Numerous publications and patents in the field describe systems based on DNA and RNA viral vectors (Kumagai *et al.*, 1994, *Proc. Natl. Acad. Sci. USA*, 90, 427-430; Mallory *et al.*, 2002, *Nature Biotechnol.* 20, 622-625; Mor *et al.*, 2003, *Biotechnol. Bioeng.*, 81; 430-437; US5316931; US5589367; US5866785; US5491076; US5977438; US5981236; WO02088369; WO02097080; WO9854342). The existing viral vector systems are usually restricted to a narrow host range in terms of their best performance and even the expression level of such vectors in their most favourable host is far below the upper biological limits of the system.

RNA viruses are the most suitable for use as expression vectors, as they offer a higher expression level compared to DNA viruses. There are several published patents which describe viral vectors suitable for systemic expression of transgenic material in plants (US5316931; US5589367; US5866785). In general, these vectors can express a foreign gene as a translational fusion with a viral protein (US5491076; US5977438), from an additional subgenomic promoter (US5466788; US5670353; US5866785), or from polycistronic viral RNA using IRES elements for independent protein translation (WO0229068). The first approach - translational fusion of a recombinant protein with a viral structural protein (Hamamoto *et al.*, 1993, *BioTechnology*, 11, 930-932; Gopinath *et al.*, 2000, *Virology*, 267, 159-173; JP6169789; US5977438) gives significant yield. However, the

use of such an approach is limited, as the recombinant protein cannot be easily separated from the viral one. One of the versions of this approach employs the translational fusion via a peptide sequence recognized by a viral site-specific protease or via a catalytic peptide (Dolja *et al.,* 1992, *Proc. Natl. Acad. Sci. USA,* 89, 10208-10212; Gopinath *et al.,* 2000, *Virology,* 267, 159-173; US5162601; US5766885; US5491076).

Expression processes utilizing viral vectors built on heterologous subgenomic promoters provide a good level of protein production (US5316931). The most serious disadvantage of such vectors and many others is their limited capacity with regard to the size of DNA to be amplified. Usually, stable constructs accommodate inserts of not more than one kb. In some areas of plant functional genomics this may not be such a serious limitation as G. della-Cioppa et al. (WO993651) described the use of TMV-based viral vectors to express plant cDNA libraries with the purpose of silencing endogenous genes. Additionally, as such vectors are capable of systemic movement and produce coat protein, significant resources of the plant are diverted from the synthesis of recombinant protein. The low expression levels achieved so far with such plant viral expression systems are a major reason why these systems are hardly competitive with other expression systems like bacterial, fungal, or insect cell expression systems. Low expression levels give rise to very high downstream costs for protein isolation and purification in a huge background of plant material. Therefore, costs for downstream processing quickly decrease, as the yield of the protein or product of interest per unit plant biomass increases. Also, a biological safety of such vectors are an issue, as they are able to form infectious viral particles.

An alternative two-component system requiring a helper virus was developed by Turpen (US 5 811,653; US 5,889,191; US 5,965,794); this approach relies on a system of a virus and a helper virus, whereby the helper virus provides a replicase function, whereas the main replicon is deficient in replicase activity. This system is not practical because viral RNA-dependent RNA polymerase (replicase) works inefficiently with substrate RNAs provided *in trans.* A possible explanation of such inefficiency is that TMV RNA-dependent RNA polymerase is a heterodimer consisting of a 126 kDa protein and a 183 kDa read-through protein (Watanabe *et al.,* 1999, *J. Virol.,* 73, 2633-2640). It was shown that at least one component of this heterodimer, the 126 kDa protein, appeared to work primarily *in cis* (Lewandowsky & Dawson, 2000, *Virology,* 271, 90-98). There are several publications concerning the complementation *in trans* of other viral functions, like cell-to cell and systemic movement. Unlike in the case of RNA dependent RNA polymerase, these functions are efficiently complemented *in trans* by 30 kDa movement protein (MP) and coat protein (CP), respectively. The MP and CP can be provided *in trans* either by a transgenic host or by another virus. For example, mutants of TMV with frameshifts within the MP or CP gene were

unable to locally or systemically infect inoculated tobacco plants, but acquired the lost functions in transgenic tobacco plants expressing the wild-type MP or CP gene (Holt & Beachy, 1991, *Virology*, 181, 109-117; Osbourn, Sarkar & Wilson, 1990, *Virology*, 179, 921-925). These works did not address the issue of creating virus-based vectors for expressing a heterologous sequence of interest, but rather studied the biological functions of different viral proteins. Another work describes the complementation of long distance movement of a CP-deficient TMV expressing GFP by a chimaeric TMV carrying ORF3 of groundnut rosette umbravirus (GRV) (Ryabov, Robinson & Taliansky, 1999, *Proc. Natl. Acad.Sci. USA*, 96, 1212-12170). However, as it follows from the results, the efficiency of GFP expression in systemic leaves of plants co-infected with CP-deficient TMV expressing GFP and TMV having CP replaced by ORF3 of GRV was significantly lower than in plants infected with systemic TMV expressing GFP. It appears that this low expression level may be due to the presence and competition of both viral vectors in systemic leaves. Moreover, all experiments mentioned above led to the formation of infectious viral particles in systemic leaves and are therefore not acceptable for use in the environment from the point of view of biological safety.

Another system proposed by C. Masuta et al. (US 5,304,731) proposes to use a satellite CMV RNA virus to be used as a carrier of the heterologous sequence of interest, and a helper virus that provides functions necessary for CMV RNA replication. To the best of our knowledge, the system is highly inefficient.

A serious concern with prior art virus-based plant expression systems is biological safety. On the one hand, high infectivity of the recombinant virus is highly desired in order to facilitate spread of the virus throughout the plant and to neighbouring plants, thereby increasing the yield of the desired gene product. On the other hand, such a high infectivity compromises containment of the recombinant material since spread to undesired plants can easily occur. Consequently, safer virus-based plant expression systems are highly desired.

There is presently no biologically safe large-scale transgene expression system built on plant RNA viral vectors capable of moving systemically and providing for the yield and efficiency required for technical applications. The existing systemic vectors suffer from a low yield of recombinant product.

Therefore, it is an object of this invention to provide an environmentally safe plant viral expression system for high-yield production of a protein of interest. It is another object of the invention to provide a process of replicating and/or expressing a nucleotide sequence of interest in a plant or plant part, which is of improved ecological and biological safety.

GENERAL DESCRIPTION OF THE INVENTION

The above objects are solved by a system for replicating or for replicating and expressing a sequence of interest in a plant, comprising:

(i) an RNA replicon or a precursor thereof, said RNA replicon being derived from a plus-sense single stranded RNA virus and comprising at least one sequence of interest; and

(ii) a helper replicon, or a precursor thereof, wherein said helper replicon is

(a) incapable of systemic movement in said plant both in the presence and in the absence of said RNA replicon (i) and

(b) capable of expressing in a plant one or more proteins necessary for systemic movement of said RNA replicon (i),

whereby said RNA replicon (i) is capable of replicating or replicating and expressing said sequence of interest in said plant, but unable to move systemically in said plant in the absence of said one or more proteins expressed by said helper replicon (ii).

The invention further provides a process of replicating or of replicating and expressing a sequence of interest in a plant, comprising providing cells of said plant with said RNA replicon (i) and said helper replicon (ii). The invention may be used for replicating a sequence of interest in a plant or for replicating a sequence of interest and expressing said sequence of interest, e.g. for producing a protein of interest like an industrial enzyme or a pharmaceutical protein in said plant. The invention also relates to proteins produced or producible by the process of the invention.

The inventors have surprisingly identified a novel principle for replicating or for replicating and expressing a sequence of interest in a plant. The inventors have found that a helper replicon that is deficient in the ability to assemble virus particles, e.g. when provided to a plant by agrodelivery as a DNA copy, can express a sufficient amount of coat protein in locally transfected tissue to fully restore systemic movement of the said RNA replicon (i). It was further found that systemic movement of said RNA replicon (i) results in systemically infected leaves that express the sequence of interest but have a greatly reduced amount of coat protein compared with the case where a coat protein-expressing virus expresses a sequence of interest. The coat protein is usually the strongest expressed protein in virus-infected plant cells. With the system and process of the invention, however, resources of systemically infected cells are not used up by coat protein expression. Consequently, the expression levels of said sequence of interest in systemically infected plant cells are higher than in conventional viral expression systems. Further, systemically infected plant cells produce small amounts of assembled viral particles from said RNA replicon (i), whereby

spread of the RNA replicon (i) to secondary host plants occurs with very low probability. If spreading of said RNA replicon (i) to an undesired plant occurs in a rare event, it cannot move systemically in such an undesired plant due to the absence of said helper replicon and poses therefore a negligible environmental risk. Thus, the system and process of the invention are of excellent biological/environmental safety. At the same time, the system and process of the invention maintain the important feature of viral expression systems that infection of a part of a plant is sufficient to achieve replication or replication and expression of a sequence of interest in other parts of the plant, preferably in the whole plant.

The advantageous features of the invention may be summarized as follows:

1. The system provides for systemic infection of the host plant by an RNA replicon that does not have a coat protein, and thus can accommodate larger DNA inserts.
2. The expression in systemic leaves is totally dedicated to the heterologous sequence of interest, and there is no or little competition with the expression of coat protein.
3. Because of the low amount of viral proteins (coat protein is present in low amounts as it is produced by the helper replicon in the locally infected leaf) and of the host proteins (due to shut off of the biosynthetic machinery of plant cell), the highest absolute and relative yield of protein of interest or RNA of interest can be achieved.
4. The yield of assembled viral particles is very low, and the assembled virus particles have no protein(s) necessary for systemic movement, thus the expression system is much more safe, than a vector that retains all functions of the wild type virus.

Further modifications of the RNA replicon (i) and of the helper replicon (ii) are described herein that minimize the risk of wild type virus reconstruction due to recombination between said RNA replicon (i) and said helper replicon (ii). Further, the invention has no detectable limit of the size of the sequence of interest to be expressed, it allows expressing multiple genes in the same cell and plant and it possesses high ecological and biological safety parameters.

The system and process of the invention can be used for replicating or for replicating and expressing a sequence of interest. Replicating refers to RNA production, namely amplification of said sequence of interest together with said RNA replicon (i). Expressing refers to the production of a protein of interest encoded in said sequence of interest. Preferably, the system and process of the invention is used for producing a protein of interest from a sequence of interest present in said RNA replicon (i).

A first component of the system (or kit) of the invention comprises said RNA replicon (i). Said RNA replicon (i) is typically derived from a plus-sense single stranded RNA virus. Examples of such viruses are cowpea mosaic virus, potato virus X, and alfalfa mosaic virus.

Preferred viruses are tobamoviruses, the most preferred ones being tobacco mosaic virus (TMV) and crucifer-infecting tobamovirus. Being derived from a plus-sense single stranded RNA virus means that said RNA replicon (i) is typically created using such a virus as a starting material. Alternatively, said RNA replicon (i) may be created using genetic functions (e.g. replicase, movement protein) from such a virus. Said RNA replicon (i) can also be created using components or genetic functions from different plus-sense single stranded RNA viruses. For being a replicon, said RNA replicon (i) has to be capable of replicating autonomously in a plant cell. Autonomous replication means that the replicon codes for a replicase (RNA-dependent RNA polymerase) catalyzing its own replication. A replicon may make use of functions of the host cell like the translation machinery needed for translating said replicase. Said replicase may be provided with one or several introns, notably if said RNA replicon (i) is provided as a DNA precursor to plant cell nuclei, for increasing the efficiency of RNA replicon build-up in the cytoplasm (cf. PCT/EP03/12530).

Further, said RNA replicon (i) contains said sequence of interest to be replicated or expressed. Preferably, said sequence of interest is expressed in the process of the invention to produce a protein of interest. Said sequence of interest is preferably heterologous to said plus-sense single-stranded RNA virus(es) from which said RNA replicon (i) is derived. Said RNA replicon (i) generally contains further genetic functions needed for expressing or replicating said sequence of interest like one or more subgenomic promoters, ribosome binding sites etc.

Said RNA replicon (i) is unable to move systemically in said plant in the absence of said one or more proteins expressed by said helper replicon (ii). This property can be achieved by modifying the nucleotide sequence encoding the protein necessary for systemic movement of said RNA virus from which said RNA replicon (i) is derived such that this protein cannot be expressed in a functional form from said RNA replicon (i). Expression in a functional form may be prevented by mutating or deleting parts of the sequence coding for said protein, or by mutating or deleting regulatory sequences (e.g. a subgenomic promoter) required for expressing said protein. In a preferred embodiment, said protein (or said proteins if said RNA virus contains more than one protein needed for systemic movement) is largely or totally deleted. Such a deletion adds several additional advantages to the system and process of the invention: Larger sequences of interest can be included in said RNA replicon (i) without compromising the efficiency of said RNA replicon (i), whereby said sequence of interest may be larger than 1 kb. Further, the homology between said RNA replicon (i) and said helper replicon (ii) is reduced. Thereby, recombination events between said replicons (i) and (ii) are unlikely.

In many plant viruses like tobamoviruses, said protein necessary for systemic movement is the coat protein. Thus, said RNA replicon (i) preferably does not contain a coat protein open reading frame (ORF) or lacks substantial parts of a coat protein ORF. Instead, said sequence of interest may take the position of the coat protein ORF. This position is 3'-proximal in many plant viruses, which is generally the position of the viral ORF that is expressed the strongest.

The second component of the system and process of the invention is said helper replicon (ii). Said helper replicon (ii) helps said RNA replicon (i) in that it is capable of expressing in a plant one or more proteins necessary for systemic movement of said RNA replicon (i). The helper replicon (ii) may provide in said plant any protein or proteins that enable(s) said RNA replicon (i) to move systemically in said plant. Preferably, said protein necessary for systemic movement corresponds to that deleted or rendered unexpressible in said RNA replicon (i). Most preferably, it is a coat protein.

Said helper replicon (ii) may be a DNA replicon and may be derived from a DNA virus like gemini virus. Preferably, however, said helper replicon (ii) is an RNA replicon and may be derived from an RNA virus, like a plus-sense single stranded RNA virus. Said helper replicon (ii) and said RNA replicon (i) may be derived from the same or from different plant viruses, e.g. from a tobamovirus like tobacco mosaic virus. Similarly as said RNA replicon (i), said helper replicon (ii) codes for a replicase catalyzing its replication in plant cells.

Said helper replicon (ii) is incapable of systemic movement in said plant independent of whether said RNA replicon (i) is present or absent in said plant. Being incapable of systemic movement may be achieved in various ways. In one embodiment, said helper replicon (ii) and said protein necessary for systemic movement are incompatible such that said protein cannot provide said helper replicon with the functionality of systemic movement. In this case, said RNA replicon (i) and said helper replicon (ii) are preferably derived from different plant viruses, and the helper replicon (ii) may be capable of expressing a coat protein that provides said RNA replicon (i) but not said helper replicon (ii) with the systemic movement functionality. Alternatively, said RNA replicon (i) and said helper replicon (ii) are derived from the same plant virus.

In a preferred embodiment, however, said helper replicon (ii) is incapable of systemic movement due to lacking a functional origin of viral particle assembly. Thereby, the helper replicon (ii) cannot be packaged by said protein necessary for systemic movement, notably said coat protein. The origin of viral particle assembly may be rendered dysfunctional. In TMV, the origin of viral particle assembly is located in the movement protein (MP) ORF. The origin of viral particle assembly in the MP ORF may also be deleted. It is not required that the

MP ORF of said helper replicon (ii) codes for a functional MP. If a functional MP protein for said helper replicon (ii) is desired, the MP protein may for example be provided by said RNA replicon (i); further, the MP may also be encoded by a plant host transgenic for MP.

Whether said helper replicon (ii) is capable or incapable of systemic movement can be tested experimentally (cf. examples) by infecting a portion of a leaf of a plant with said replicon (or a precursor thereof) and observing the occurrence of the same replicon in other, non-infected, leaves ("systemic leaves") of this plant. Being incapable of systemic movement is a relative property. Said helper replicon (ii) is considered to be incapable of systemic movement if the probability of systemic movement is substantially reduced compared to the virus it is derived from. In any case, the probability of systemic movement of said helper replicon (ii) is considerably lower than that of said RNA replicon (i), such that replication or replication and expression of said sequence of interest from said RNA replicon (i) in systemic leaves is not suppressed in systemic leaves in the typical time-frame of protein expression with plant viral expression systems (about 1 to 3 weeks). Most preferably, no systemic movement of said helper replicon (ii) in said plant is detectable (e.g. by Western or Northern blotting).

For improving the environmental safety and the efficiency of the system, said RNA replicon (i) and said helper replicon (ii) should not be prone to recombination with each other. This may be achieved by having a low homology between said replicons (i) and (ii). Preferably, said RNA replicon (i) and said helper replicon (ii) lack homology in functionally overlapping regions or do not overlap. Functionally overlapping regions are regions in said replicons having or coding for the same function, e.g. the replicase ORF, the MP ORF, or subgenomic promoters. Homology in such regions may be reduced e.g. by changing codons using the degeneracy of the genetic code and/or by using functional regions for said replicon (i) and (ii) that derive from different plant viruses.

Many potential recombination events do not change said replicons or lead to unfunctional replicons, depending on the specific replicons. Such recombinations may reduce the efficiency of the system but do not pose an environmental risk. For achieving the best environmental safety, said RNA replicon (i) and said helper replicon (ii) preferably lack a recombination-prone homology in a region, wherein recombination between said RNA replicon (i) and said helper replicon (ii) would create a replicon capable of, at the same time,

(A) expressing a protein necessary for systemic movement and

(B) moving systemically in a plant.

Such a replicon would be comparable to viral vectors in conventional plant viral expression systems. The skilled person can easily identify such regions. In an embodiment wherein both

replicons (i) and (ii) are based on tobamoviruses and said helper replicon (ii) lacks a functional origin of viral particle assembly, such a region is that downstream of the location in the MP ORF where the origin of assembly was deleted or rendered unfunctional. In this region, the homology between said RNA replicon (i) and said helper replicon (ii) should be reduced. Since in TMV the 3' part of the MP ORF and the 5' part of the CP subgenomic promoter overlap and in some strains the MP ORF further contains a part of the CP ORF, the possibilities of changing the codon usage is limited for reducing the homology, since this may impair the function of the CP subgenomic promoter. Instead of changing the codon usage, functional replacement may be used, i.e. the use of functional elements that derive from different viruses like a heterologous subgenomic promoter.

The system of the invention thus contains at least said components (i) and (ii). The system of the invention may contain said RNA replicon (i) and/or said helper replicon (ii) in the form of a precursor from which said replicons (i) and (ii) are formed in cells of the plant. The precursor of said RNA replicon (i) will generally be DNA coding for said RNA replicon (i) and having a promoter functional in said plant for forming said RNA replicon (i) by transcription of said DNA in cells of said plant. Similarly, if said helper replicon (ii) is an RNA replicon (RNA replicon (ii)), the precursor of said helper replicon (ii) may be DNA. Said DNA precursors may be flanked by T-DNA left and right border sequences and may be carried by agrobacteria. In a particularly preferred embodiment of the invention, said system of the invention comprises, in an Agrobacterium-carried T-DNA, a DNA precursor of said RNA replicon (i); and, in an Agrobacterium-carried T-DNA, a DNA precursor of said helper replicon (ii). Other precursors of said replicons (i) and (ii) for said system of the invention are DNA for biolistic transformation of said plant or for other transformation methods.

The system of the invention may further contain a plant or seeds thereof for carrying out the process of the invention. In principal, the invention may be carried out with any plant for which infectious viruses are known. Preferred are crop plants including monocot and dicot plants, whereby the latter are preferred. The invention is well-established with *Nicotiana* plants and may be applied to other plants of the family *Solanaceae*. *Nicotiana tabacum* and *N. benthamiana* are most preferred. These plants have the additional advantage that they typically do not enter the human food chain.

Said plant may be a wild-type plant or a transgenic plant. An MP gene stably and expressibly integrated in the genome of said plant may be used for complementing the MP function of said helper replicon (ii), as described above. A preferred MP for this purpose is the MP of tobacco mosaic virus.

Obviously, said plant used for the process of the invention, said RNA replicon (i), and said helper replicon (ii) have to be appropriately selected for giving a functional system. For example, said replicons (i) and (ii) have to be able to replicate in cells of said plants, the used MPs have to be functional for enabling cell-to-cell movement in said plant, the used coat protein has to able to provide systemic movement to said RNA replicon (i) in said plant, etc. These issues are familiar to people of skill in the art.

In the process of the invention, said RNA replicon (i) and said helper replicon (ii), or precursors thereof, are provided to cells of said plant. Said replicons (i) and (ii) may be provided directly to said plant, which may be as RNA molecules or as packaged viral particles. Alternatively, said RNA replicon (i) or said helper replicon (ii) or both said RNA replicon (i) and said helper replicon (ii) may be provided to said plant as DNA precursors of said RNA replicon (i) and/or said helper replicon (ii). The type of precursor depends on the type of transformation method to be used. Usable transformation methods are given below. A preferred transformation method is *Agrobacterium*-mediated transformation. In this case, said plant is provided with said RNA replicon (i) and/or said helper replicon (ii) by transfecting with agrobacteria containing in their T-DNA said precursor of said replicon (i) and/or with agrobacteria containing in their T-DNA said precursor of said helper replicon (ii).

When said plant is transformed with said RNA replicon (i) and said helper replicon (ii), or precursors thereof, a selected part of said plant should be treated with both replicons in order to allow complementation of said RNA replicon (i) by said helper replicon (ii). In the case of *Agrobacterium*-mediated transformation, this is most easily achieved by treating a selected part of said plant with a mixture of two *Agrobacterium* strains, one strain containing said RNA replicon (i) as a DNA precursor in T-DNA, and another strain containing said helper replicon (ii) as a DNA precursor in T-DNA. Since at least one of said replicons (i) and (ii) will generally be capable of cell-to-cell movement due to an MP, it is not absolutely required that a cell of said plants are transformed with both replicons (i) and (ii). For reasons of efficiency, it is, however, preferred that cells of said plant are transformed with both replicons (i) and (ii).

For making full use of the invention, selected parts of said plant like one or more leaves, preferably lower leaves, should be provided with said RNA replicon (i) and said helper replicon (ii) but not other parts of said plant. Other parts of said plants, notably systemic leaves, will then be reached by said RNA replicon (i) by way of systemic movement.

The plants that can be used for the process of the invention correspond to those that may be a component of the system of the invention.

Said replicated or expressed sequence of interest may be harvested from said plant by conventional means. These products may be isolated using the whole plant, i.e. plant

material that was provided with said replicons (i) and (ii) and plant material that was not provided with said replicons (i) and (ii). Preferably, these products are harvested and isolated from plant parts that were not provided with said replicons (i) and (ii), like leaves systemically infected by said RNA replicon (i).

PREFERRED EMBODIMENTS

A system for replicating or for replicating and expressing a sequence of interest in a plant, comprising:

(i) Agrobacteria containing a T-DNA comprising a precursor of an RNA replicon, whereby said RNA replicon is derived from tobacco mosaic virus, lacks a functional coat protein coding sequence, and comprises at least one sequence of interest; and

(ii) Agrobacteria containing a T-DNA comprising a precursor of a helper replicon derived from a tobamovirus, wherein said helper replicon

  (a) lacks a functional origin of viral particle assembly and is incapable of systemic movement in said plant and

  (b) is capable of expressing in said plant a tobacco mosaic virus coat protein necessary for systemic movement of said RNA replicon (i),

whereby said RNA replicon (i) is capable of replicating or replicating and expressing said sequence of interest in said plant, but unable to move systemically in said plant in the absence of said tobacco mosaic virus coat protein expressed by said helper replicon (ii).

A process of expressing a sequence of interest in a *Nicotiana* plant, comprising co-transforming a leaf of said plant with a mixture of the Agrobacteria of the above system.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts schemes of T-DNA regions for vectors pICH8543, pICH17272, pICH10595, pICH16601, pICH17501, pICH16684, pICH17344 designed for having an increased frequency of RNA virus-based replicon formation in plant cells. Some constructs contain introns which are numbered. The numbers correspond to the introns given in the appendix. pICH8543, pICH17272, and pICH17344 are RNA replicons (i) according to the invention. pICH16601 and pICH16684 are helper replicons (ii) according to the invention.

Act2 – promoter of the Arabidopsis ACTIN2 gene; RdRp - viral RNA-dependent RNA polymerase; MP – viral movement protein; NTR – viral 3' non-translated region; CP – viral coat protein; Tnos – transcription termination region of nopalin synthase.

Fig. 2 shows systemic *N. benthamiana* leaves of two plants co-infitrated with pICH17272 and pICH16684.

Fig. 3 shows an *N. benthamiana* plant co-infiltrated with pICH17272 and pICH17501. The left picture shows, under UV-light, the infiltrated area circled in the picture on the right hand side. GFP expression is found exclusively in the co-infiltrated area.

Fig. 4 shows SDS gel electrophoretic separation (coomassie stained) of total soluble proteins extracted from infiltrated and systemic leaves of *N. benthamiana.*

Lanes: 1. pICH16601 upper non-infiltrated leaf tissue
2. pICH8543 infiltrated area
3. Molecular weight marker
4. Systemic leaf of a plant infected with TVCV
5,6. systemic leaf of plant co-infiltrated with pICH16684 and pICH17272
7. systemic leaf of a plant co-infiltrated with pICH16601 and pICH17272
8. Molecular weight marker
9. pICH8543 infiltrated area
10. Systemic leaf of a plant infected with TVCV
11. pICH16601 upper non-infiltrated leaf tissue
12 to 14. pICH17344, systemic leaf

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a process of highly efficient and biologically safe systemic expression of a sequence or protein of interest using an RNA virus-derived replicon (said RNA replicon (i)). This process overcomes limitations of existing RNA viral vector-based expression systems, such as size limitation for heterologous sequences to be expressed systemically and high instability of said vectors. Further, said process offers better biosafety characteristics and prevents the formation of wild type viruses due to recombination of viral components. The replicons (i) and (ii) of the invention can be designed such that such recombinations are avoided. The approach described herein allows for a rapid and highly efficient expression of a sequence of interest in a whole plant including systemic leaves.

To our knowledge, there are no efficient two-component systems relying on the use of (a) vectors that are deficient in systemic movement (lacking functional coat protein) and (b) transgenic plants providing the missing coat protein in trans. Such systems are not practically useful, probably because the levels of coat protein expressed even under a strong constitutive promoter is insufficient to provide for efficient systemic movement of the vector. In addition, creation of transgenic plants is time consuming and should be avoided in those applications where rapid expression of small amounts of protein or RNA of interest is required. Also, in case where a constitutive promoter provides for the expression of a sufficient amount of coat protein to support systemic movement, the biological safety of the system would be low, as the assembly of infectious viral particles would take place in systemic leaves.

Many different RNA viruses belonging to different taxonomic groups are suitable for constructing said RNA replicon (i) and said helper replicon (ii) of this invention, subject to the identification of the viral elements responsible for systemic movement and to the possibility to reconstitute the systemic movement functionality of the RNA replicon (i) by expressing one of such elements in trans from the helper replicon (ii) of the invention. A list of RNA viruses which can be used for the creation of said RNA replicon (i) and said helper replicon (ii) of this invention is presented below. Taxa names in quotes (and not in italic script) indicate that this taxon does not have an ICTV international approved name. Species (vernacular) names are given in regular script. Viruses with no formal assignment to genus or family are indicated:

RNA Viruses:

ssRNA Viruses: Family: *Bromoviridae*, Genus: *Alfamovirus*, ***Type species:*** alfalfa mosaic virus, **Genus:** *Ilarvirus*, ***Type species:*** tobacco streak virus, **Genus:** *Bromovirus*,

Type species: brome mosaic virus, Genus: Cucumovirus, **Type species:** cucumber mosaic virus;

Family: *Closteroviridae,* **Genus:** *Closterovirus*, ***Type species:*** beet yellows virus, **Genus:** *Crinivirus*, ***Type species:*** Lettuce infectious yellows virus, **Family:** *Comoviridae*, **Genus:** *Comovirus, **Type species:** cowpea mosaic virus,* **Genus:** *Fabavirus, **Type species:*** broad bean wilt virus 1, **Genus:** *Nepovirus*, ***Type species:*** tobacco ringspot virus;

Family: *Potyviridae,* **Genus:** *Potyvirus*, ***Type species:*** potato virus Y, **Genus:** *Rymovirus*, ***Type species:*** *ryegrass mosaic virus*, **Genus:** *Bymovirus*, ***Type species:*** barley yellow mosaic virus;

Family: *Sequiviridae*, **Genus:** *Sequivirus*, ***Type species:*** parsnip yellow fleck virus, **Genus:** ***Waikavirus*, *Type species:*** rice tungro spherical virus; **Family:** *Tombusviridae*, **Genus:** *Carmovirus, **Type species:*** carnation mottle virus, **Genus:** *Dianthovirus*, ***Type species***: carnation ringspot virus, **Genus:** *Machlomovirus, **Type species:*** maize chlorotic mottle virus, **Genus:** *Necrovirus, **Type species***: tobacco necrosis virus, **Genus:** *Tombusvirus, **Type species***: tomato bushy stunt virus, **Unassigned Genera of ssRNA viruses, Genus:** *Capillovirus*, ***Type species:*** apple stem grooving virus;

Genus: *Carlavirus*, ***Type species:*** carnation latent virus; **Genus:** *Enamovirus*, ***Type species:*** pea enation mosaic virus,

Genus: *Furovirus, **Type species:*** soil-borne wheat mosaic virus, **Genus:** *Hordeivirus*, ***Type species:*** barley stripe mosaic virus, **Genus:** *Idaeovirus, **Type species:*** raspberry bushy dwarf virus;

Genus: *Luteovirus, **Type species:*** barley yellow dwarf virus; **Genus:** *Marafivirus*, ***Type species:*** maize rayado fino virus; **Genus:** *Potexvirus*, ***Type species:*** potato virus X;**Genus:** *Sobemovirus, **Type species:*** Southern bean mosaic virus, **Genus:** *Tenuivirus*, ***Type species:*** **rice stripe virus,**

Genus: *Tobamovirus*, ***Type species:*** tobacco mosaic virus,

Genus: *Tobravirus, **Type species:*** tobacco rattle virus,

Genus: *Trichovirus, **Type species:*** apple chlorotic leaf spot virus; **Genus:** *Tymovirus*, ***Type species:*** turnip yellow mosaic virus; **Genus:** *Umbravirus*, ***Type species:*** carrot mottle virus;

In addition to TMV-based expression systems, viral vectors for expressing heterologous genes of interest were developed on the basis of several other plus sense ssRNA viruses, such as potato virus X (Mallory *et al.*, 2002, *Nat. Biotechnol.*, 20, 622-625),

alfalfa mosaic virus (Sanches-Navarro *et al.*, 2001, *Arch. Virol.*, 146, 923-939), and cowpea mosaic virus (Gopinas *et al.*, 2000, 267, 159-173). The strategy described in this invention for TMV-based vectors can also be employed to the viral expression systems mentioned above.

The construction of different types of TMV-based viral vectors used in this invention (Fig. 1) is described in examples 1 to 5. Vector pICH8543 (example 1) has an origin of (viral particle) assembly but lacks a coat protein (CP) coding sequence. This vector and its intron-containing derivative pICH17272 (example 4) are capable of cell-to-cell movement and sequence of interest (GFP) expression in primary infected leaves and contain an origin of assembly, but are unable to move systemically due to the absence of a coat protein. Another pair of vectors, pICH10595 (example 2) and pICH17501 (example 5), encode, in addition to MP, the CP instead of GFP, and are able to move systemically and to form infectious viral particles. Co-infiltration of the vectors pICH17272 and pICH17501 (Fig. 3) does not lead to GFP expression in systemic leaves. GFP is strongly expressed only in the primary inoculated leaf, but viral symptoms are clearly visible in systemic leaves. The explanation of this result is that an expression vector without CP (pICH17272) cannot compete with a helper virus (pICH17501) capable of moving systemically.

In order to address this problem, the helper viral vectors (helper replicon (ii)) pICH16601 and pICH16684 (Fig. 1, example 3) that are capable of expressing CP but lack the origin of assembly and are consequently unable to move systemically were generated. Co-infiltration of these helper viral vectors with RNA replicons (i) that express GFP but are unable to move systemically leads to the appearance of GFP in systemic leaves (Fig. 2). The total soluble protein from systemic leaves of plants infected with the two-vector system (Fig. 4, lanes 5-7) contained a high level of GFP that was comparable to that of primary inoculated leaves (Fig. 4, lane 2). This high expression level is not achieved when a systemically moving viral vector is used for GFP expression, as such a viral vector predominantly expresses CP (Fig. 4, lanes 12-14).

A minor amount of CP is also produced in systemic leaves by the two-component system of the invention (Fig. 4, lane 7). This can be explained by the presence of recombined viral vector capable of expressing CP and of moving systemically. However, the relative proportion of such recombinants is negligible and does not have any significant impact on the productivity (expression level) described above. In addition, the frequency of such recombinations can be further reduced and even eliminated completely, by reducing the length of overlapping stretches or the homology between the helper replicon (ii) and the RNA replicon (i) that expresses the sequence of interest.

The reduction or complete elimination of homologous functional regions being targets for homologous recombination can be achieved by several different approaches: deletions of said regions; changes of coding regions within the regions of homology by applying different codon usage; changing said regions by directed evolution (for review of approach see Tobin *et al.*, 2000, *Curr. Opin. Struct. Biol.*, 10, 421-427). In case of TMV–based viral vector systems, one can use different RNA-dependent RNA polymerases (RdRp) for the RNA replicon (i) that expresses said sequence of interest and the helper replicon (ii). Well characterised RdRps for this purpose are e.g. the RdRps of TVCV, TMV-U1, TMV-U5, or crTMV.

In our specific experiments, the homology within RdRp coding regions does not cause a serious recombination problem as the only recombination region within the short region of homology is located at the 3' end of the MP gene in front of GFP in case of the RNA replicon (i) (see Fig.1, plasmids pICH8543; pICH17272) and the part of the MP coding sequence located in front of the CP ORF of the helper replicon (ii) (Fig. 1, pICH16601; pICH16684) might lead to the formation of wild type virus-like replicons compromising the efficiency of the system. Such short regions can be easily modified by various methods in order to remove the homology and any chance of undesired recombination events. In addition, certain functions of the replicons (i) and/or replicon (ii) like MP expression necessary for cell-to-cell movement, may be provided *in trans* by a transgenic host plant (Holt & Beachy, 1991, *Virology*, 181, 109-117). In such an embodiment, only those parts of the MP ORF overlapping with the RdRp of said helper replicon and the origin of assembly can be left in said RNA replicon (i). In general, there are many different strategies to reduce or completely remove the recombination frequency between said RNA replicon (i) and said helper replicon (ii), that can be easily performed by a person familiar with the art.

Different methods may be used for providing cells of a plant with said RNA replicon (i) and/or said helper replicon (ii). Said vectors may be transformed into plant cells by a Ti-plasmid vector carried by *Agrobacterium* (US 5,591,616; US 4,940,838; US 5,464,763) or particle or microprojectile bombardment (US 05100792; EP 00444882B1; EP 00434616B1). Other plant transformation methods can also be used like microinjection (WO 09209696; WO 09400583A1; EP 175966B1), electroporation (EP00564595B1; EP00290395B1; WO 08706614A1) or PEG-mediated transformation of protoplasts etc. The choice of the method for vector delivery depends on the plant species to be transformed and the vector used. For example, microprojectile bombardment is generally preferred for vector delivery in monocot, while for dicots, *Agrobacterium*-mediated transformation gives better results in general.

In the examples described below, we used *Agrobacterium*-mediated delivery of vectors into *Nicotiana* cells. However, the vectors may be introduced into the plants in

accordance with any of the standard techniques suitable for stable or transient transformation of the plant species of interest. Transformation techniques for dicotyledonous are well known in the art and include *Agrobacterium*-based techniques and techniques which do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. These techniques include PEG or electroporation mediated uptake, particle bombardment-mediated delivery and microinjection. Examples of these techniques are described in Paszkowski *et al.*, *EMBO J* 3, 2717-2722 (1984), Potrykus *et al.*, *Mol. Gen. Genet.* 199,169-177 (1985), Reich *et al.*, Biotechnology *4*:1001-1004 (1986), and Klein *et al.*, *Nature* 327,70-73 (1987). In each case, the transformed cells are regenerated to whole plants using standard techniques.

Agrobacterium-mediated transformation is a preferred technique for the transformation of dicotyledons because of its high transformation efficiency and its broad utility with many different plant species. The many crop species which may be routinely transformed by *Agrobacterium* include tobacco, tomato, sunflower, cotton, oilseed rape, potato, soybean, alfalfa and poplar (EP 0 317 511 (cotton), EP 0 249 432 (tomato), WO 87/07299 (*Brassica*), U.S. Patent 4,795,855 (poplar)).

In the examples of this invention, we used agro-inoculation, a method of *Agrobacterium*-mediated delivery of T-DNA for transient expression of gene(s) of interest (Vaquero *et al.*, 1999, *Proc. Natl. Acad. Sci. USA*, 96, 11128-11133). Agro-inoculation is an extremely useful tool not only for small-to-middle scale recombinant protein production systems, but as one of the elements of a vector optimisation system allowing to obtain fast results with different variants of constructs.

This invention is not limited to TMV-based vectors described in examples 1 - 5, but can be extended to replicons based on other plant RNA viruses.

Sequences or genes of interest, their fragments (functional or non-functional) and their artificial derivatives that can be expressed in plants or plants cells using the present invention include, but are not limited to: starch modifying enzymes (starch synthase, starch phosphorylation enzyme, debranching enzyme, starch branching enzyme, starch branching enzyme II, granule bound starch synthase), sucrose phosphate synthase, sucrose phosphorylase, polygalacturonase, polyfructan sucrase, ADP glucose pyrophosphorylase, cyclodextrin glycosyltransferase, fructosyl transferase, glycogen synthase, pectin esterase, aprotinin, avidin, bacterial levansucrase, *E.coli* glgA protein, MAPK4 and orthologues, nitrogen assimilation/methabolism enzyme, glutamine synthase, plant osmotin, 2S albumin, thaumatin, site-specific recombinase/integrase (FLP, Cre, R recombinase, Int, SSVI Integrase R, Integrase phiC31, or an active fragment or variant thereof), oil modifying

enzymes (like fatty acids desaturases, elongases etc), isopentenyl transferase, Sca M5 (soybean calmodulin), coleopteran type toxin or an insecticidally active fragment, ubiquitin conjugating enzyme (E2) fusion proteins, enzymes that metabolise lipids, amino acids, sugars, nucleic acids and polysaccharides, superoxide dismutase, inactive proenzyme form of a protease, plant protein toxins, traits altering fiber in fiber producing plants, Coleopteran active toxin from *Bacillus thuringiensis* (Bt2 toxin, insecticidal crystal protein (ICP), CrylC toxin, delta endotoxin, polyopeptide toxin, protoxin etc.), insect specific toxin AaIT, cellulose degrading enzymes, E1 cellulase from *Acidothermus celluloticus*, lignin modifying enzymes, cinnamoyl alcohol dehydrogenase, trehalose-6-phosphate synthase, enzymes of cytokinin metabolic pathway, HMG-CoA reductase, *E. coli* inorganic pyrophosphatase, seed storage protein, *Erwinia herbicola* lycopen synthase, ACC oxidase, pTOM36 encoded protein, phytase, ketohydrolase, acetoacetyl CoA reductase, PHB (polyhydroxybutanoate) synthase, enzymes involved in the synthesis of polyhydroxylalkanoates (PHA), acyl carrier protein, napin, EA9, non-higher plant phytoene synthase, pTOM5 encoded protein, ETR (ethylene receptor), plastidic pyruvate phosphate dikinase, nematode-inducible transmembrane pore protein, trait enhancing photosynthetic or plastid function of the plant cell, stilbene synthase, an enzyme capable of hydroxylating phenols, catechol dioxygenase, catechol 2,3-dioxygenase, chloromuconate cycloisomerase, anthranilate synthase, *Brassica* AGL15 protein, fructose 1,6-biphosphatase (FBPase), AMV RNA3, PVY replicase, PLRV replicase, potyvirus coat protein, CMV coat protein, TMV coat protein, luteovirus replicase, MDMV messenger RNA, mutant geminiviral replicase, *Umbellularia californica* C12:0 preferring acyl-ACP thioesterase, plant C10 or C12:0 preferring acyl-ACP thioesterase, C14:0 preferring acyl-ACP thioesterase (luxD), plant synthase factor A, plant synthase factor B, D6-desaturase, proteins having an enzymatic activity in fatty acids biosynthesis and modifications, e.g. the peroxysomal □-oxidation of fatty acids in plant cells, acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, lipase, maize acetyl-CoA-carboxylase, etc.; 5-enolpyruvylshikimate-3-phosphate synthase (EPSP), phosphinothricin acetyl transferase (BAR, PAT), CP4 protein, ACC deaminase, protein having posttranslational cleavage site, DHPS gene conferring sulfonamide resistance, bacterial nitrilase, 2,4-D monooxygenase, acetolactate synthase or acetohydroxyacid synthase (ALS, AHAS), polygalacturonase, Taq polymerase, bacterial nitrilase, many other enzymes of bacterial or phage origin including restriction endonucleases, methylases, DNA and RNA ligases, DNA and RNA polymerases, reverse trascryptases, nucleases (Dnases and RNAses), phosphatases, transferases etc.

The present invention can be used for the purpose of molecular farming and purification of commercially valuable and pharmaceutically important proteins including

industrial enzymes (cellulases, lipases, proteases, phytases etc.) and fibrous proteins (collagen, spider silk protein, etc.). Human or animal health protein may be expressed and purified using described in our invention approach. Examples of such proteins of interest include inter alia immune response proteins (monoclonal antibodies, single chain antibodies, T cell receptors etc.), antigens including those derived from pathogenic microorganisms, colony stimulating factors, relaxins, polypeptide hormones including somatotropin (HGH) and proinsulin, cytokines and their receptors, interferons, growth factors and coagulation factors, enzymatically active lysosomal enzyme, fibrinolytic polypeptides, blood clotting factors, trypsinogen, a1-antitrypsin (AAT), human serum albumin, glucocerebrosidases, native cholera toxin B as well as function-conservative proteins like fusions, mutant versions and synthetic derivatives of the above proteins.

EXAMPLES

Information on the genetics of tobamoviruses like TMV and crucifer-infecting tobamovirus can be found in WO02/029068.

EXAMPLE 1

Construction of a GFP-expressing TMV-based RNA vector

A cr-TMV-based viral vector containing GFP, pICH8543 (Fig. 1), has been described in international patent application PCT/EP03/12530 (see also below). This clone contains the *Arabidopsis* Actin2 promoter, the TVCV RNA-dependent RNA polymerase, a chimaeric sequence (TVCV/cr-TMV) for the movement protein, the GFP coding sequence, the 3' untranslated region of cr-TMV and finally the Nos terminator, cloned in a binary vector. This clone lacks a coat protein coding sequence. pICH8543 was transformed into *Agrobacterium* strain GV3101 and infiltrated into one leaf of a *Nicotiana benthamiana* plant using a needle-less syringe. Four days after infiltration, GFP fluorescence foci could be seen in the infiltrated area. Fluorescence lasted for several weeks in the infiltrated leaf but did not move to upper uninoculated leaves.

Construction of vector pICH8543

A replicon containing a green fluorescence protein (GFP) gene was made in several cloning steps. The resulting construct, pICH8543, contains in sequential order: a 787 bp fragment from the *Arabidopsis* actin 2 promoter (*ACT2*, ref An et al, 1996, GenBank accession AB026654, bp 57962 to 58748), the 5' end of TVCV (GenBank accession

BRU03387, bp 1 to 5455), a fragment of cr-TMV (GenBank accession Z29370, bp 5457 to 5677, with thymine 5606 changed to cytosine to remove the start codon of the coat protein, CP), sequences "taa tcg ata act cga g", a synthetic GFP (sGFP) gene, cr-TMV 3' nontranslated region (3' NTR; GenBank accession Z29370, bp 6078 to 6312), and finally the nopaline synthase (Nos) terminator. The entire fragment was cloned between the T-DNA left (LB) and right (RB) borders of pICBV10, a $Carb^R$ pBIN19-derived binary vector.

EXAMPLE 2

Construction of a CP-expressing TMV-based RNA vector

Cloned cDNAs of the crucifer-infecting tobamovirus (cr-TMV; Dorokhov *et al.*, 1994, *FEBS Lett.* 350, 5-8) and of the turnip vein-clearing virus (TVCV; Lartey *et al.*, 1994, *Arch. Virol.* 138, 287-298) were obtained from Prof. Atabekov from Moscow University, Russia. A viral vector expressing TVCV CP was made by subcloning an EcoRI-ApaI fragment (containing part of MP, the complete CP coding sequence, and the 3' non translated region of TVCV) from the TVCV cDNA into pICH8543. The resulting clone, pICH10595 (Fig. 1), contains the complete TVCV cDNA cloned between the *Arabidopsis* Actin2 promoter and the Nos terminator, in a binary vector. The pICH10595 was transformed in *Agrobacterium* strain GV3101 and infiltrated into a *Nicotiana benthamiana* leaf. Three weeks later, the upper non-infiltrated leaves had a wrinkled yellow appearance indicating viral infection. Polyacrylamide gel electrophoresis (PAGE) with coomassie staining and Western blot analysis revealed that the systemic leaves expressed large amounts of coat protein, indicating that pICH10595 is functional.

To test whether CP produced by pICH10595 could package *in trans* the RNA replicon of pICH8543, both clones were co-infiltrated into a *Nicotiana benthamiana* leaf. Five days after infiltration, GFP could be detected in the inoculated leaf area. However, no GFP could be detected in upper leaves, even three weeks after infiltration. At this time, the inoculated plant had typical symptoms of a plant inoculated with wild-type virus: it was shorter than a control uninoculated plant, and upper leaves were wrinkled and yellowish. PAGE and coomassie staining of the gel showed that CP was expressed in the systemic leaf, but no GFP protein could be detected. Western blot analysis also failed to detect GFP in the systemic leaves, although GFP was detected in the inoculated leaf. In conclusion, a wildtype virus is unable to move a viral amplicon expressing a gene of interest (such as GFP) *in trans.*

EXAMPLE 3

Construction of a CP-expressing clone lacking an origin of assembly

In the previous example, only the wild type virus is detected in the upper uninoculated leaves when the lower leaves are first infiltrated with a mixture of CP and GFP-expressing clones. The CP clone is so efficient to replicate and move that the GFP-expressing clone cannot compete effectively and move systemically. To prevent the CP-expressing clone to move systemically, we removed the area that putatively contains the origin of assembly (OAS) of the virus (the CP-expressing clone), which corresponds to part of the MP located upstream of the CP subgenomic promoter. The resulting clone, pICH16601 (Fig. 1) is similar to pICH10595 but lacks base pairs 4966-5454 (coordinates relative to GenBank accession BRU03387). pICH16601 was transformed in *Agrobacterium* strain GV3101 and was coinfiltrated with pICH8543 into a *Nicotiana benthamiana* leaf. Twelve days later, GFP fluorescence appeared in the veins of the upper non-infiltrated leaves. Then, on the following days, GFP fluorescence grew out of the veins into leaf tissue and covered part of the leaf area. PAGE and coomassie staining as well as Western blot analysis showed that GFP protein was made in the systemic leaves. Therefore, removing the OAS from the CP-expressing clone allows a clone of interest to move systemically by providing CP *in trans.*

EXAMPLE 4

Addition of introns to the construct containing the gene of interest improves its systemic movement

To improve the efficiency of coexpression of the CP-expressing clone and of the clone of interest in the infiltrated area, introns were inserted in the RdRp and the MP of the construct containing the heterologous sequence of interest. pICH8543 was modified by addition of ten *Arabidopsis* introns in the RdRp (introns 1 to 10, sequence in appendix) at positions 1844, 2228, 2588, 2944, 3143, 3381, 3672, 3850, 4299, 4497 (coordinates relative to GenBank accession BRU03387) and of two introns in the MP (introns 11-12) at positions 5287 and 5444. The resulting clone, pICH17272 (Fig. 1) was coinfiltrated in an *Nicotiana benthamiana* leaf with pICH16601. GFP appeared in the systemic leaves starting 7 days after infiltration, faster than when a clone without intron was used.

EXAMPLE 5

Addition of introns to the CP-expressing construct improves systemic movement of the construct containing the sequence of interest.

To improve expression of CP in a larger number of cells in the infiltrated area, introns 1 to 9 (described above) were added to pICH16601, at the same positions as described for the construction of pICH17272. In addition, the MP subgenomic promoter area was replaced by less T-rich and more GC-rich sequence. As a result, the sequence between bp 4585 to 5460 (coordinates relative to GenBank accession BRU03387) were replaced by Seq 1 given in the appendix, resulting in conctruct pICH16684 (Fig 1). pICH16684 was coinfiltrated with pICH17272 in an *N. benthamiana* leaf. Seven days after infiltration, GFP appeared in systemic leaves (Fig. 2). More tissue expressing GFP was obtained than when clones without introns were used.

An additional CP-expressing construct was made by the addition of 6 introns (introns 1 to 3 and 8 to 9, as described for construction of pICH17272) to pICH10595. This construct, pICH17501 (Fig. 1), contains a complete MP and therefore contains the OAS. This construct was coinfiltrated with pICH17272. GFP was expressed in the infiltrated leaf area, but never move to upper systemic leaves (Fig. 3). Only CP-expressing amplicons were found in the systemic leaf. This shows that the presence of introns is not sufficient for systemic movement, and that removing the OAS from the CP-expressing clone is essential.

EXAMPLE 6

The gene of interest is expressed at high level in systemic leaf

To compare the approach described herein with systemic movement obtained using traditional vectors which express CP from the same molecule as the gene of interest, we made a control construct, pICH17344, that expresses both GFP and CP (Fig. 1). This construct is similar to pICH17272 but the 3' end of the 3'NTR (corresponding to bp 6274 to 6312 in GenBank accession Z29370) was replaced by the 1005 3' terminal sequence from tobacco mild green mosaic virus (TMGMV) U5 variant (bp 5498 to 6502). pICH17344 was transformed in *Agrobacterium* GV3101 and infiltrated into a *Nicotiana benthamiana* leaf. Seven days after inoculation, GFP fluorescence was detected in systemic leaves. Analysis by PAGE and coommassie staining showed that more CP was made than GFP in the systemic leaves of plants inoculated with pICH17344 (Fig. 4). In contrast, no or little CP was present in the systemic leaves of plants inoculated with a mixture of pICH16684 and pICH17272 (Fig. 4). Therefore most expressed protein in systemic leaves corresponds to the gene of interest instead of CP.

Some CP is made in systemic leaves of plants inoculated with a mixture of pICH16684 and pICH17272, albeit less than with pICH17344. This most likely occurs as a result of recombination between pICH16684 and pICH17272, producing a wildtype CP-expressing virus. Reducing homology between both clones by making a CP-expressing clone based on a related but different virus (for example TMV strain U1) can reduce or eliminate such recombination events.

Appendix

intron 1

gtaaatcctggtccacacttttacgataaaaacacaagattttaaactatgaactgatcaataatcattcctaaaagaccacacttttg
ttttgtttctaaagtaatttttactgttataacag

intron 2

gtaagaggtcaaaaggtttccgcaatgatccctctttttttgtttctctagtttcaagaatttgggtatatgactaacttctgagtgttccttg
atgcatatttgtgatgagacaaatgtttgttctatgttttag

intron 3

gtaagttctgcatttggttatgctccttgcattttaggtgttcgtcgctcttccatttccatgaatagctaagatttttttttctctgcattcattcttc
ttgcctcagttctaactgtttgtggtatttttgttttaattattgctacag

intron 4

gtaaagcaactgtgttttaatcaatttcttgtcaggatatatggattataacttaatttttgagaaatctgtagtatttggcgtgaaatgagt
ttgctttttggtttctcccgtgttatag

intron 5

gtaaagtttccaactttcctttaccatatcaaactaaagttcgaaacttttttatttgatcaacttcaaggccacccgatctttctattcctga
ttaatttgtgatgaatccatattgacttttgatggttacgcag

intron 6

gtctgtctttcctatttcatatgtttaatcctaggaatttgatcaattgattgtatgtatgtcgatcccaagactttcttgttcacttatatcttaa
ctctctctttgctgtttcttgcag

intron 7

gtaaaatattggatgccagacgatattctttcttttgatttgtaacttttcctgtcaaggtcgataaattttatttttttttggtaaaaggtcgat
aatttttttttggagccattatgtaattttcctaattaactgaaccaaaattatacaaaccag

intron 8

gtaaggacttctcatgaatattagtggcagattagtgttgttaaagtctttggttagataatcgatgcctcctaattgtccatgttttactgg
ttttctacaattaaag

intron 9

gtgagttcctaagttccatttttttgtaatccttcaatgttattttaacttttcagatcaacatcaaaattaggttcaattttcatcaaccaaat
aatatttttcatgtatatatag

intron 10

gtaagtttttccactttaagaaaattactagcactaaatttacgaattttaactatacaattatggatgtaaccaccattttaaattaatctt
gaaccagacgatatggattacaaacattcttgttttaatcggctggttagctattgcatttgcag

intron 11

gtaaggatttttatgatatagtatgcttatgtattttgtactgaaagcatatcctgcttcattgggatattactgaaagcatttaactacatgt
aaactcacttgatgatcaataaacttgattttgcag

intron 12

gtaagccatcttcctgcttatttttataatgaacatagaaataggaagttgtgcagagaaactaattaacctgactcaaaatctaccct
cataattgttgtttgatattggtcttgtattttgcag

Seq1

cggacgatacgtgatccaccatgatagaggagccattgtgtattacgatccgcttaaactaatatctaagctcggctgcaagcaca
tcagagacgtcgtgcacttagaagagttacgcgagtctttgtgcgacgtagctagtaacttgaacaactgcgcctacttctcacagtt
agatgaggccgttgctgaggtccacaagactgcggtcggaggctccttcgcgttctgtagcatcatcaaatacttgtcagacaaga
ggctgttcagggacctgttcttcgtctgagttgacgaattc

Claims

1. A system for replicating or for replicating and expressing a sequence of interest in a plant, comprising:
   (i) an RNA replicon or a precursor thereof, said RNA replicon being derived from a plus-sense single stranded RNA virus and comprising at least one sequence of interest; and
   (ii) a helper replicon, or a precursor thereof, wherein said helper replicon is
      (a) incapable of systemic movement in said plant both in the presence and in the absence of said RNA replicon (i) and
      (b) capable of expressing in a plant one or more proteins necessary for systemic movement of said RNA replicon (i),
   whereby said RNA replicon (i) is capable of replicating or replicating and expressing said sequence of interest in said plant, but unable to move systemically in said plant in the absence of said one or more proteins expressed by said helper replicon (ii).

2. The system according to claim 1, wherein said helper replicon (ii) is incapable of systemic movement in a plant due to the absence of a functional origin of viral particle assembly.

3. The system according to claim 1 or 2, wherein said helper replicon (ii) is capable of expressing in a plant a coat protein and/or a movement protein necessary or useful for said systemic movement of said RNA replicon (i) in said plant.

4. The system according to any one of claims 1 to 3, wherein said RNA replicon (i) cannot express a coat protein necessary for systemic movement of said RNA replicon (i) in the plant.

5. The system according to claim 4, wherein said RNA replicon (i) lacks a coat protein open reading frame and said sequence of interest is larger than 1 kb.

6. The system according to any one of the preceding claims, wherein said RNA replicon (i) is based on, or contains components of, a tobamovirus.

7. The system according to claim 6, wherein said tobamovirus is a tobacco mosaic virus.

8. The system according to claim 6 or 7, wherein said RNA replicon (i) is based on a

tobamovirus wherein the coat protein open reading frame is replaced by said sequence of interest.

9. The system according to any one of claims 1 to 5, wherein said RNA replicon (i) is based on or contains components of alfalfa mosaic virus.

10. The system according to any one of claims 1 to 5, wherein said RNA replicon (i) is based on or contains components of potato virus X.

11. The system according to any one of claims 1 to 5, wherein said RNA replicon (i) is based on or contains components of cowpea mosaic virus.

12. The system according to any one of claims 1 to 11, wherein said precursor of said RNA replicon (i) is DNA encoding said RNA replicon (i), and said DNA is capable of producing said RNA replicon (i) in cells of said plant.

13. The system according to claim 12, wherein said precursor of said RNA replicon (i) contains one or more introns; or was modified relative to the virus it is derived from by changing the codon usage.

14. The system according to any one of claims 1 to 13, wherein said precursor of said helper replicon (ii) is DNA encoding said helper replicon (ii), whereby said DNA is capable of producing said helper replicon (ii) in cells of said plant.

15. The system according to any one of the preceding claims, wherein said precursor of said RNA replicon (i) or said precursor of said helper replicon (ii) are carried by agrobacteria.

16. The system according to any one of the preceding claims, wherein the system further comprises a plant, or seeds thereof, for replicating or for replicating and expressing said sequence of interest.

17. The system according to any one of claims 1 to 16, wherein said plant is a dicot plant, preferably a *Solanacea* plant, more preferably a *Nicotiana* plant, most preferably tobacco.

18. The system according to any one of claims 1 to 17, wherein said plant is transgenic

and expresses a viral protein necessary or useful for cell-to-cell movement of said RNA replicon (i) or said helper replicon (ii).

19. The system according to claim 18, wherein said viral protein is a movement protein of tobacco mosaic virus.

20. The system according to any one of the preceding claims, wherein said RNA replicon (i) and said helper replicon (ii) lack homology in functionally overlapping regions.

21. The system according to any one of the preceding claims, wherein said RNA replicon (i) and said helper replicon (ii) lack a recombination-prone homology in a region recombination in which between said RNA replicon (i) and said helper replicon (ii) would create an RNA replicon capable of
    (A) expressing a protein necessary for systemic movement and
    (B) moving systemically in said plant.

22. The system according to any one of the preceding claims, wherein the sequence of said RNA replicon (i) and the sequence of said helper replicon (ii) do not overlap.

23. A process of replicating or for replicating and expressing a sequence of interest in a plant, comprising providing cells of a plant with
    (i) an RNA replicon or a precursor thereof, said RNA replicon being derived from a plus-sense single stranded RNA virus and comprising at least one sequence of interest; and
    (ii) a helper replicon, or a precursor thereof, wherein said helper replicon is
        (a) incapable of systemic movement in a plant both in the presence and in the absence of said RNA replicon (i) and
        (b) capable of expressing in a plant one or more proteins necessary for systemic movement of said RNA replicon,

    whereby said RNA replicon (i) is capable of replicating or of replicating and expressing said sequence of interest in a plant, but unable to move systemically in a plant in the absence of said one or more proteins expressed by said helper replicon (ii).

24. The process according to claim 23, wherein said RNA replicon (i) or said helper replicon (ii) or both said RNA replicon (i) and said helper replicon (ii) are provided to said plant as DNA precursors of said RNA replicon (i) and/or said helper replicon (ii).

25. The process according to claim 23 or 24, wherein said plant is provided with said RNA replicon (i) and/or said helper replicon (ii) by transfecting with agrobacteria containing in their T-DNA said precursor of said replicon (i) and/or with agrobacteria containing in their T-DNA said precursor of said helper replicon (ii).

26. The process according to any one of claims 23 to 25, wherein a part of said plant like a leaf is provided with said RNA replicon (i) and said helper replicon (ii) but not other parts of said plant.

27. The process according to any one of claims 23 to 26, wherein said sequence of interest is capable of replicating or of replicating and expressing systemically in parts of said plant not provided with both said RNA replicon (i) and said helper replicon (ii).

28. The process according to any one of claims 23 to 27, wherein said plant is a dicot plant, preferably a *Solanacea* plant, more preferably a *Nicotiana* plant, most preferably said plant is tobacco.

29. Use of the system according to any one of claims 1 to 22 for expressing a protein of interest in a plant from said sequence of interest.

Abstract

A system for replicating or for replicating and expressing a sequence of interest in a plant, comprising:

(i) an RNA replicon or a precursor thereof, said RNA replicon being derived from a plus-sense single stranded RNA virus and comprising at least one sequence of interest; and

(ii) a helper replicon, or a precursor thereof, wherein said helper replicon is

(a) incapable of systemic movement in said plant both in the presence and in the absence of said RNA replicon (i) and

(b) capable of expressing in a plant one or more proteins necessary for systemic movement of said RNA replicon (i),

whereby said RNA replicon (i) is capable of replicating or replicating and expressing said sequence of interest in said plant, but unable to move systemically in said plant in the absence of said one or more proteins expressed by said helper replicon (ii).

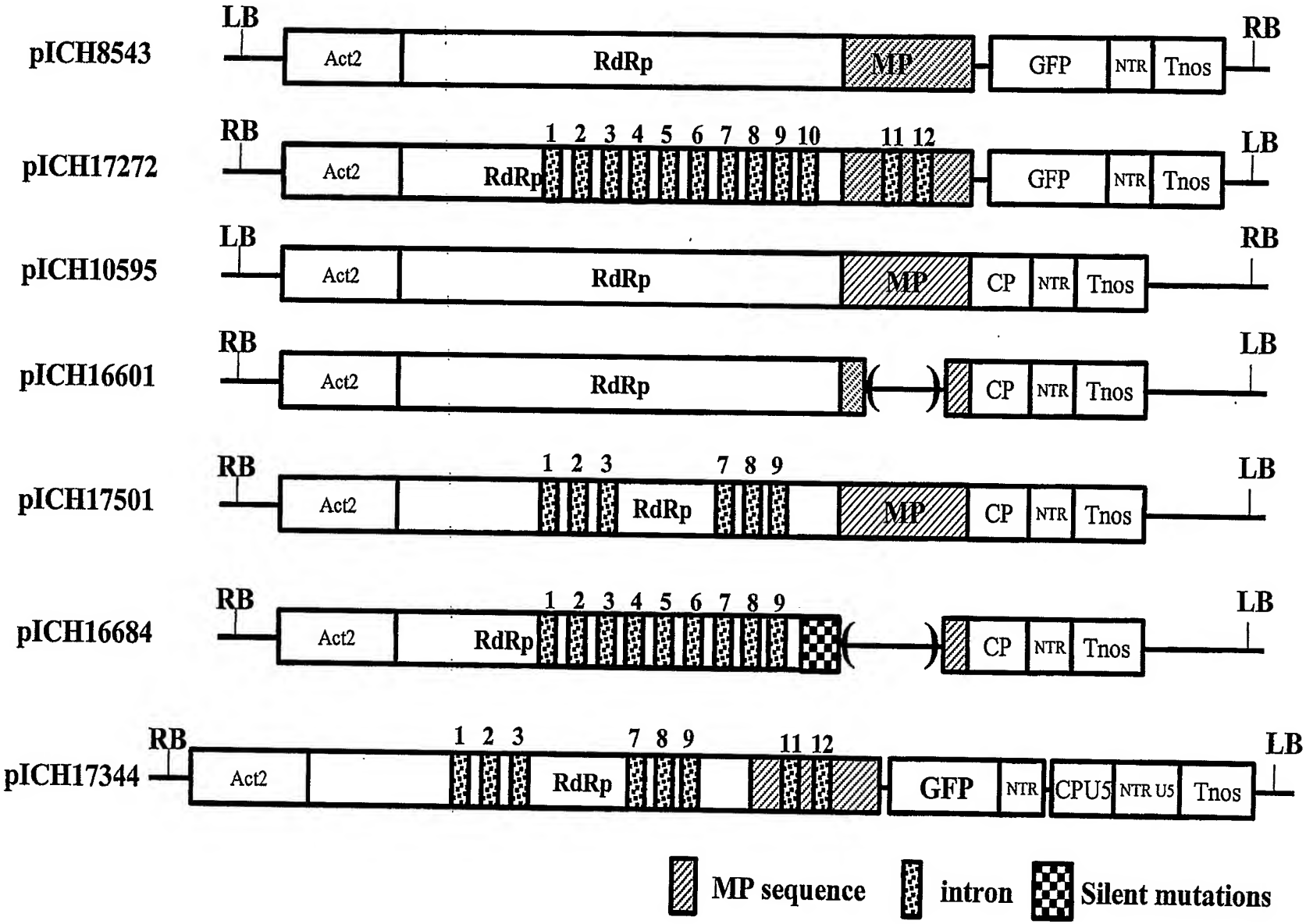

Fig. 1

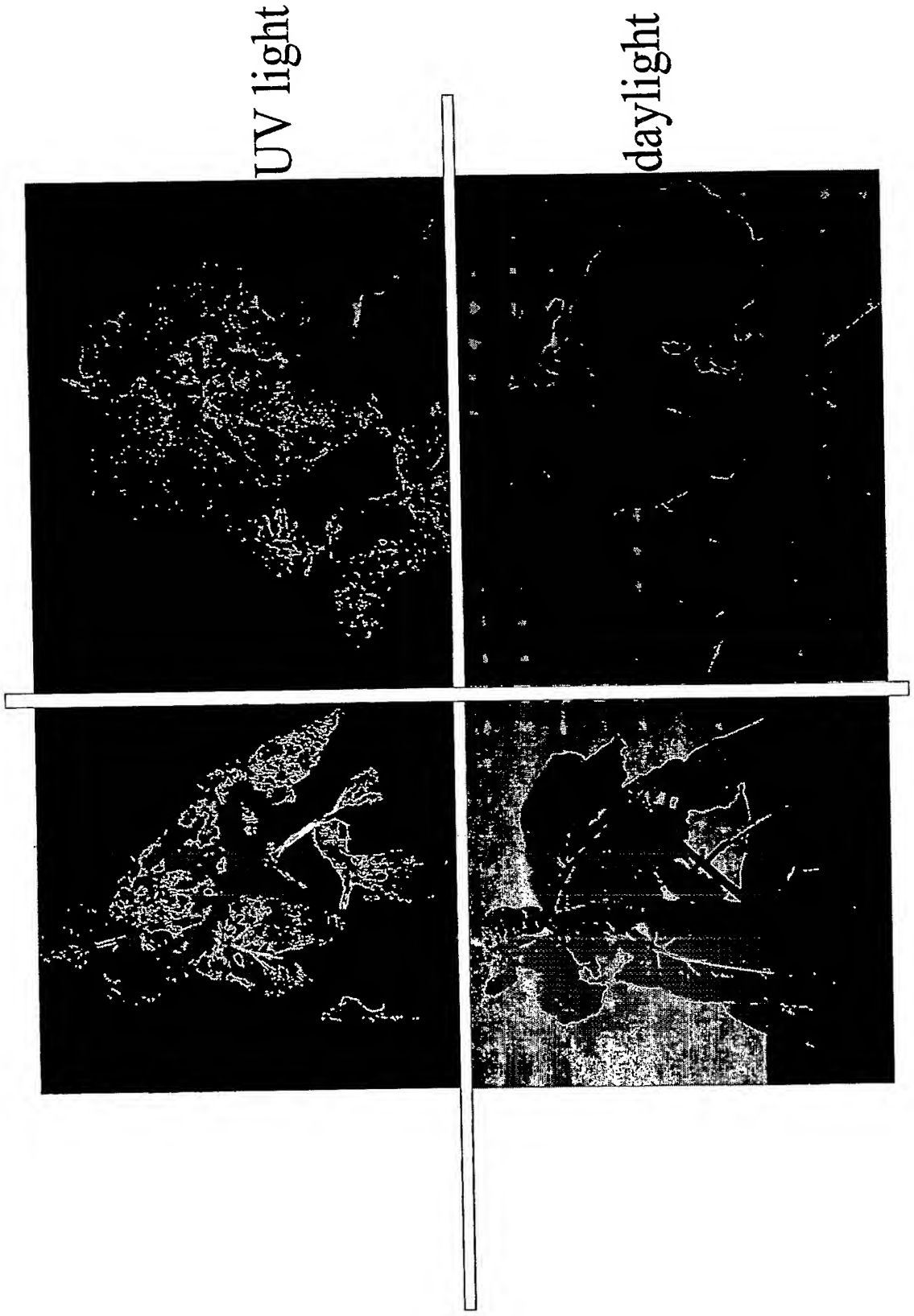

Fig. 2

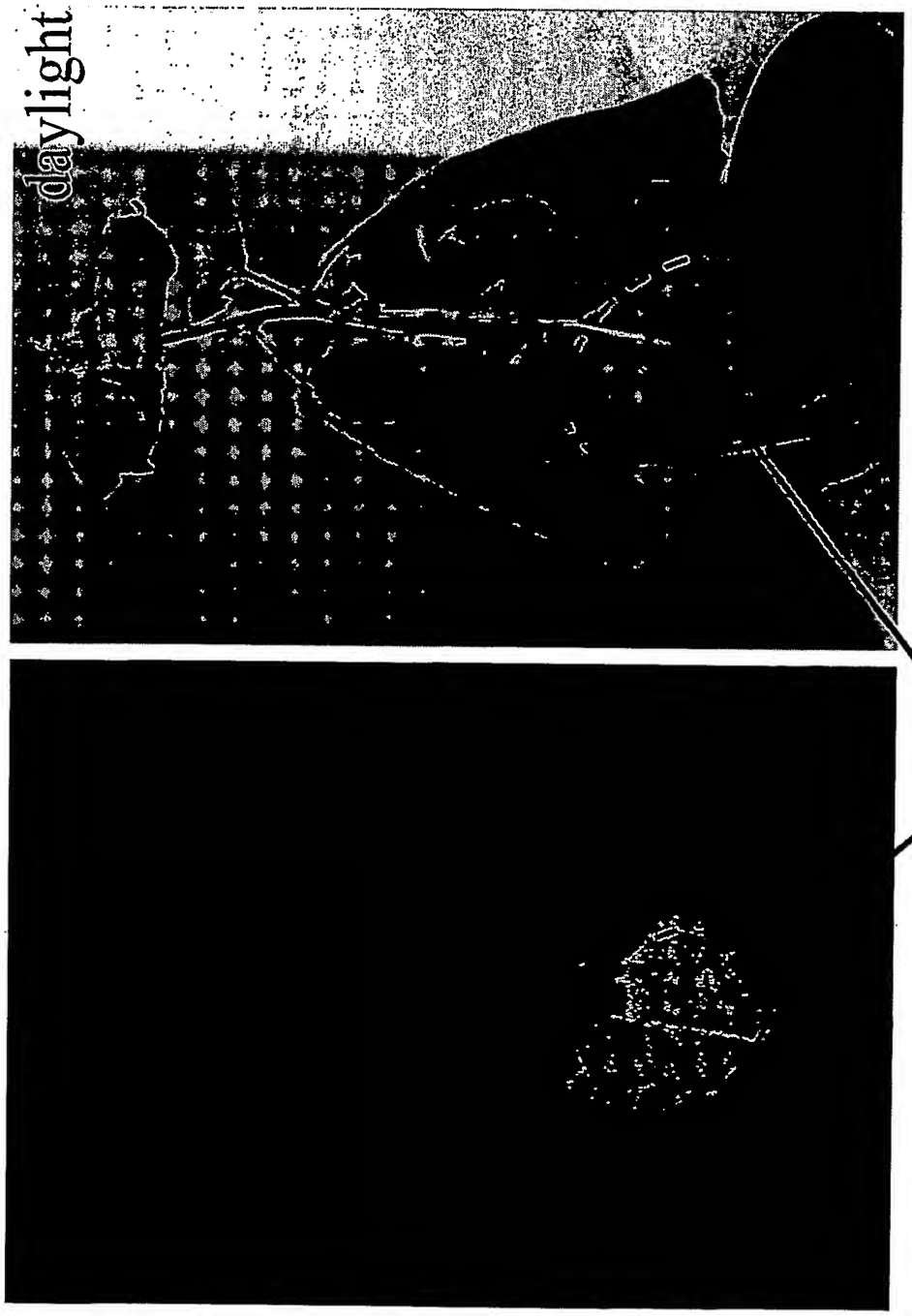

Fig. 3

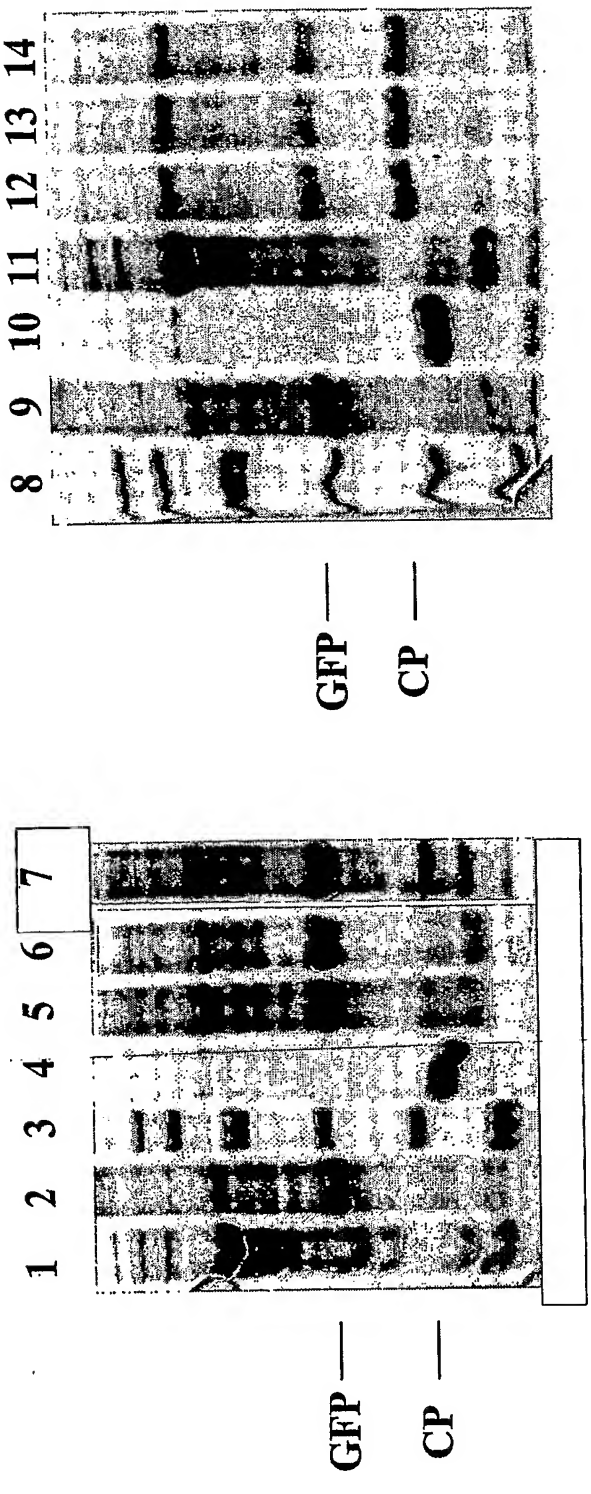

Fig. 4